Modeling neuronal defects associated with a lysosomal disorder using patient-derived induced pluripotent stem cells

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By providing access to affected neurons, human induced pluripotent stem cells (iPSc) offer a unique opportunity to model human neurodegenerative diseases. We generated human iPSc from the skin fibroblasts of children with mucopolysaccharidosis type IIIB. In this fatal lysosomal storage disease, defective $\alpha$-N-acetyl-galactosaminidase interrupts the degradation of heparan sulfate (HS) proteoglycans and induces cell disorders predominating in the central nervous system, causing relentless progression toward severe mental retardation. Partially digested proteoglycans, which affect fibroblast growth factor signaling, accumulated in patient cells. They impaired isolation of emerging iPSc unless exogenous supply of the missing enzyme cleared storage and restored cell proliferation. After several passages, patient iPSc starved of an exogenous enzyme continued to proliferate in the presence of fibroblast growth factor despite HS accumulation. Survival and neural differentiation of patient iPSc were comparable with unaffected controls. Whereas cell pathology was modest in floating neurosphere cultures, undifferentiated patient iPSc and their neuronal progeny expressed cell disorders consisting of storage vesicles and severe disorganization of Golgi ribbons associated with modified expression of the Golgi matrix protein GM130. Gene expression profiling in neural stem cells pointed to alterations of extracellular matrix constituents and cell–matrix interactions, whereas genes associated with lysosome or Golgi apparatus functions were downregulated. Taken together, these results suggest defective responses of patient undifferentiated stem cells and neurons to environmental cues, which possibly affect Golgi organization, cell migration and neuritogenesis. This could have potential consequences on post-natal neurological development, once HS proteoglycan accumulation becomes prominent in the affected child brain.

INTRODUCTION

Although neurodegenerative disorders are mostly prevalent in the elderly, they also occur early in life. In infants and children, inborn errors of metabolism, in particular lysosomal storage diseases (LSDs), represent the most frequent cause of neurodegeneration. LSDs result from inefficient recycling of macromolecule catabolites, which accumulate and cause a multitude of clinical symptoms, including progressive mental retardation.

Whereas primary genetic and biochemical disorders are well described in LSDs, the limitations of currently available study models hampered accurate exploration of downstream cascades of events. Investigations in mouse models or in
patient cell cultures identified disorders consistent with cell autonomous dysfunctions. They affect endocytosis (1,2), macro-autophagy (3), calcium homeostasis (4,5), mitochondria (6), endoplasmic reticulum homeostasis (7,8), phospholipid synthesis (9,10), neurite outgrowth (5,11) and neural stem cell production, migration and differentiation (12). In the central nervous system (CNS), neural cell disorders are associated with neuroinflammation, which presumably aggravates neurodegeneration (13). However, the relevance of cell disorders to the neurological manifestations observed in children remains unclear.

Mucopolysaccharidosis IIIb (MPSIIIB, Sanfilippo syndrome type B) is an LSD caused by α-N-acetylgalactosaminidase (NAGLU, EC 3.2.1.50) deficiency, a lysosomal hydrolase involved in heparan sulfate (HS) proteoglycan degradation. Severe neurological manifestations contrast with mild expression of the disease in peripheral organs. The onset of behavioral changes is at 3–5 years of age, followed by progressive mental retardation and premature death (14). Patient autopsy revealed storage lesions in the CNS, macrophages, liver and kidney epithelial cells, but not in other tissues (15–17). Similar pathology was observed in the mouse model of the disease (18,19). Predominance of pathology in the CNS emphasizes the importance of analyzing neural cells and neurons to elucidate pathophysiology. Intra-cellular vacuoles with characteristics of lysosomes in brain cells is the pathological hallmark of the disease. Studying these lesions in cortical neurons of the MPSIIIB mouse, we showed that they were associated with Golgi complex alterations and modified expression of the Golgi matrix protein GM130 (20), a tethering protein important for Golgi structure and dynamics (21). We did not observe disorders of endocytosis or macro-autophagy in these cells. The re-programmation of human skin fibroblasts into induced pluripotent stem cells (iPSc) and their subsequent differentiation in neural progenitors and neurons provide access to human neurons, the cell type most relevant to clinical expression of MPSIIIB in children. Reprogramming of MPSIIIB patient fibroblasts was efficient. However, the proliferation of emerging iPSc was impaired unless the enzyme defect was complemented by an exogenous enzyme. Patient undifferentiated iPSc and neurons expressed cell disorders, which were not observed in parental skin fibroblasts and in floating neurospheres. These defects may affect neurological development in young children.

**RESULTS**

**Complementation of enzymatic defect allows isolation of patient iPSc**

Skin fibroblast primary cultures were established from two MPSIIIB patients for diagnosis purpose. Patient 1 was homozygous for a previously undescribed splice donor mutation in the NAGLU gene (c.531+1G>C). Patient 2 was homozygous for p.R482W mutation in exon 6 (22). NAGLU activity was below the detection threshold in cultured fibroblast extracts (0.04 ± 0.01 and 0.03 ± 0.02 μkat/kg, respectively, versus 1.23 ± 0.02 μkat/kg in controls).

Cultured fibroblasts were exposed to retroviral vectors encoding OCT4, SOX2, KLF4 (control and patient 2), or to these three vectors combined with a vector coding for c-MYC (control and patient 1). After exposure, fibroblasts were grown on a feeder layer of irradiated mouse embryonic fibroblasts (iMEF) in the presence of basic fibroblast growth factor (FGF2, 10 ng/ml). Emerging iPSc-like clones positive for alkaline phosphatase (AP) were visible after 3–4 weeks. Equivalent clone numbers were scored in control and MPSIIIB fibroblasts (~0.1% of plated fibroblasts with the four vectors, ~0.01% without c-MYC vector).

Control iPSc-like clones rapidly expanded (cloning efficiency 87.5%, n = 24). In contrast, patient iPSc-like clones poorly proliferated and were inefficiently cloned (cloning efficiency 2.5%, n = 120; Supplementary Material, Fig. S1). Since FGFs act in concert with HS proteoglycans to activate FGF receptors (23) and partially digested HS accumulates in MPSIIIB cells, we assumed that impaired proliferation of emerging patient iPSc resulted from altered FGF2 signaling caused by HS. We therefore isolated patient iPSc-like clones in the presence of exogenous NAGLU to clear HS (24). For that purpose, genetically engineered iMEF (NAGLU-iMEF) releasing NAGLU enzyme in culture supernatant were isolated. When grown on this feeder, patient iPSc-like clones rapidly expanded and were as efficiently cloned as control iPSc (cloning efficiency 83.3%, n = 12; Supplementary Material, Fig. S1).

**Characterization of iPSc clones**

iPSc clones from control (C1, C2, C3), patient 1 (P1.1, P1.2, P1.3) and patient 2 (P2.1, P2.2, P2.3) were propagated for at least 20 passages on iMEF (control), or NAGLU-iMEF (patients). They showed normal karyotypes at passages 1–5 and at least 20 passages on iMEF (control), or NAGLU-iMEF (patients). They showed normal karyotypes at passages 15–20 (data not shown). Mutations in the NAGLU gene were retrieved in patient clones. Cells expressed markers of pluripotency (SSEA4, TRA1-60, NANOG, Fig. 1A). Quantitative PCR with reverse transcription (qRT-PCR) indicated efficient repression of exogenously introduced genes (Fig. 1B). Endogenous pluripotency-associated genes were re-activated (Fig. 1C). Whereas the OCT4 promoter was heavily methylated in parent fibroblasts, it was demethylated in iPSc clones, consistent with epigenetic reprogramming to pluripotency (Fig. 1D). Six weeks after transplantation in immunodeficient mice, all control or patient iPSc formed teratoma. Histological examination revealed tissues from the three embryonic germ layers (Fig. 1E). Thus, all control and patient iPSc clones were defined as human pluripotent iPSc.

Storage lesions are prominent in undifferentiated patient iPSc clones

Since exogenous NAGLU prevents expression of disease-related features, patient 1 and patient 2 iPSc clones were starved from the NAGLU enzyme after 15–20 passages. After transfer of patient iPSc clones to Matrigel™-coated wells in the presence of FGF2 (100 ng/ml) for 10 days, NAGLU catalytic activity was undetectable and cells were intensely stained with anti-HS antibodies (Fig. 2A).

Patient 1 and patient 2 undifferentiated iPSc clones accumulated vacuoles positive for the lysosome-associated membrane...
protein 1 (LAMP1) (Fig. 2B) and these vacuoles were highly distended (1.50 ± 0.17 μm² in patient iPSc, n = 97, versus 0.065 ± 0.04 μm² in controls, n = 88, P = 0.02). Electron microscopy revealed large vesicles with heterogeneous contents (Fig. 2D). Western blot showed higher amounts of LAMP1 in patient than in control iPSc clones (Fig. 3A), whereas LAMP1 mRNA levels were equivalent (data not shown). Additional disorders consisted of Golgi alterations in both patients’ iPSc clones with modified GM130 staining patterns (Fig. 2B) and co-staining of LAMP1-positive distended vesicles with anti-GM130 antibodies (Fig. 2C). Intense GM130 staining and expression in patient iPSc clones (Figs 2B and F and 3B) suggested increased Golgi size or GM130 reactivity outside Golgi. Moreover, Golgi structure was disorganized, consisting of fragmented ribbons, accumulating small vesicles and apposition of storage vesicles to Golgi cisterna rims (Fig. 2E). However, the vesicularization of Golgi stacks during mitosis was not affected in patients’ iPSc (Supplementary Material, Fig S2A). Both patients showed similar defects. Taken together, these results showed that undifferentiated patient iPSc exhibited prominent cell pathology. In contrast, cell pathology was absent in parental patient skin fibroblasts despite HS accumulation (Fig. 2A). LAMP1 and GM130 expressions were comparable with control fibroblasts, and Golgi complexes showed similar morphology (Supplementary Material, Fig. S3).

**Patient iPSc clones are pluripotent**

iPSc differentiation can be examined in vitro by preventing cell adhesion, which results in the formation of embryoid bodies (EBs). After 10 days of non-adherent growth,
Figure 2. Undifferentiated patient iPSc express disease-related features. All control, patient 1 and patient 2 iPSc clones were cultured for 10 days on Matrigel™-coated wells. (A) (Left panels) Immunostaining reveals the accumulation of HS proteoglycans (in green) and expression of the pluripotency marker SSEA-4 (in red) in patient iPSc (iPS-P1 Matrigel), compared with control (iPS-C Matrigel), or with patient iPSc grown in the presence of exogenous NAGLU (iPS-P1 iMEF-NAGLU). Representative images of iPSc clones are shown. Scale bar: 10 µm. (Right panels) Immunostaining shows HS proteoglycan accumulation (in green) in patient 1 (hFib-P1) and patient 2 (hFib-P2) fibroblasts compared with control (hFib-C). Scale bar: 20 µm. (B–C) iPSc cultured on Matrigel™-coated wells were immunolabeled for LAMP1 (in purple) and GM130 (in green). (B) Confocal immunofluorescence shows distended LAMP1-positive vesicles and modified GM130 staining pattern in patient iPSc (iPS-P1), compared with control (iPS-C). Representative images are shown. Scale bar: 10 µm. (C) (Upper panel) High magnification confocal view of doubly stained cells. Scale bar: 5 µm. (Lower panels) Deconvoluted images without (left) or with (right) iso-surface treatment show contiguous staining for LAMP1 and GM130 in vesicle limiting membranes. (D–E) iPSc cultured on Matrigel™-coated wells were processed for electron microscopy. (D) Low (top panels) or high (lower panels) magnification images show accumulation of enlarged intracellular vesicles with heterogeneous content in patient iPSc, but not in control iPSc. Representative images of one patient-2 iPSc clone (iPS-P2) and of one control clone (iPS-C) are shown. Similar observations were made in patient 1 iPSc clones. (E) Representative images of Golgi apparatus are shown. Normal cells (iPS-C) show Golgi ribbons with well-aligned and stacked cisternal membranes. In contrast, multiple short stacks are visible in iPS-P1, which are not linked to each other. In iPS-P2, cisternae are distended, interrupted and sometimes connected to storage vesicles (StV, arrow), N, nucleus. Scale bars: upper left panels: 1 µm; lower panels, right panels: 0.5 µm. (F) GM130 expression was quantified by flow cytometry in control (iPS-C) and patient (iPS-P) iPSc. After a 10-day culture period on Matrigel™-coated wells, dissociated iPSc were stained with an anti-GM130 antibody. An isotypic mAb was used as a negative control (black line). A representative flow cytometry diagram is shown. The bar chart on the right indicates mean ± SEM fluorescence intensity values from independent analyses of control (n = 3) or patient (n = 4) iPSc. *P < 0.05 (Mann–Whitney test).
NAGLU activity was not detectable in patient 1 and patient 2 EB extracts (Supplementary Material, Fig. S4). The catalytic activity of other enzymes was also affected, as usually observed in LSDs (Supplementary Material, Fig. S4) (25). The formation and survival of EBs were nevertheless equally efficient for all control and patient iPSc clones. After adhesion for 10 days on polyornithine–laminin-coated cover slips, EBs derived from patients’ clones accumulated enlarged intracellular LAMP1-positive vesicles (Fig. 4A). Despite both enzyme deficiency and cell pathology, iPSc differentiation was not impaired in this in vitro assay. All patient or control iPSc clones produced cells representing the three germ layers (Fig. 4B), including cells positive for MAP2 and nestin (ectoderm), α-smooth muscle actin (SMA) (mesoderm) and α-fetoprotein (AFP) (endoderm).

Neural stem cells derived from patient iPSc show modified gene expression profiles

All control, patient 1 and patient 2 iPSc clones give rise to proliferating floating neurospheres after removal from feeder layers and cultivation in the presence of FGF2 and epidermal growth factor (EGF). After 2 weeks of non-adherent growth, neurospheres expressed nestin, a marker of early neural progenitors (Fig. 5A). Patient 1 and patient 2 neurospheres accumulated glycosaminoglycan (GAG), including HS (Fig. 5B; Supplementary Material, S5). However, immunofluorescence (Fig. 5C), western blot (Fig. 3C and D) and flow cytometry analyses (Fig. 5D) did not show higher expression of LAMP1 or GM130 in patient than in control neural stem cells. Annexin assays indicated equivalent proportion of apoptotic cells in patient and control neural stem cells (Supplementary Material, Fig. S6). Electron microscopy examination of ultra-thin sections performed on neurosphere pellets revealed rare storage vacuoles, and although Golgi alterations were present, they were infrequent and milder than in undifferentiated iPSc (Fig. 5E).

With the aim to determine whether undegraded HS modify biological functions even in the absence of major visible cell pathology, we compared gene expression profiles between cells that did or did not accumulate HS. Total RNA was extracted from patient 2 parental fibroblasts before and after genetic correction of the enzyme defect, and from iPSc-derived neurosphere cultures (two controls, C1 and C3, and three patients, P1.1, P1.3, P2.3). Although only 50 mRNAs showed different levels in corrected and deficient fibroblasts (among 47 000 transcripts), NAGLU deficiency was associated with the modification of 3280 mRNAs (among 29 000 transcripts) in patient neural stem cells (microarray raw data available at http://www.ebi.ac.uk/arrayexpress/ E-MEXP-3033 and http://www.ncbi.nlm.nih.gov/geo/ GSE23075). Genes with modified expression overlapped between the two cell types (23 out of the 50 modified fibroblast transcripts, Supplementary Material, Table S1). They pointed to modifications of extra-cellular matrix (ECM) constituents (synthesis of HS chains, fibronectin, tenascin, thrombospondin, elastin, collagens III and XI, nidogen and laminin gamma, proteases and proteases inhibitor involved in matrix turnover), molecules involved in cell–matrix interactions (integrins, FGFs, FGF receptors, semaphorins, ephrin, ephrin receptor, Slit), cell adhesion (calpain, protein kinase Cβ, PDK1, phosphatidylinositol 3 kinase, tensin, parvin, RhogTPases activating proteins, guanine exchange factors and Cdc42 effectors) and pathways transducing extra-cellular signals (PAK1, mitogen-activated kinases, c-fos, c-jun, phospholipase A2). Several constituents of the TGFβ and wnt signaling pathways were also modified. Predicted functional consequences focus on cell communication (P-value 8 × 10-6), adhesion (3 × 10-4), division (9 × 10-4), motility (9 × 10-7) and axon guidance (2 × 10-7). Additional modifications detected in neural stem cells concerned developmental processes (4 × 10-21), especially mesoderm (1 × 10^{-14}) and ectoderm (8 × 10^{-10}) development, neurogenesis (2 × 10^{-10}) or angiogenesis (9 × 10^{-7}).

Expression levels of genes encoding proteins associated with lysosomes or the Golgi were modified in patients’ neural stem cells but not in patients’ fibroblasts (Supplementary Material, Table S2). With the exception of DRAM (damage-regulated autophagy modifier, P-value 2 × 10^{-3}) and CD1d (10^{-7}), all genes encoding proteins associated with lysosomes (26) were downregulated in patient cells, including lysosomal hydrolases, proton pumps, transmembrane transporters and other lysosomal membrane proteins. With the exception of GOLM1 (Golgi membrane protein 1, P-value 2 × 10^{-3}), genes associated with Golgi morphology, localization and trafficking were downregulated.

**Patient neurons show severe storage lesions and Golgi complex alterations**

We next examined neural cell defects after adhesion of patient floating neurospheres. The differentiation of cells growing as floating neurospheres was induced by dissociation and adhesion on polyornithine–laminin-coated cover slips. Control and patient cultures contained equivalent proportions of β3-tubulin-positive differentiated neurons (40.6 ± 7.6%, n = 328, and 38 ± 7.3%, n = 339, after 3 weeks, respectively, Fig. 6A), equivalent proportions of GABAergic neurons (15.6 ± 4.2%, n = 275, and 12.3 ± 2.4%, n = 224, of the MAP2-positive neuron population after 5 weeks, respectively,
Fig. 6B), equivalent ratios between GABAergic and glutamatergic neurons. Synaptophysin puncta outlining MAP2-positive neurons suggested efficient neuronal maturation (Fig. 6B). GFAP-positive cells represented <0.1% of differentiated cells. Cells positive for the oligodendrocytic markers O4 or NG2 were not observed. We did not observe differences in the kinetics, efficiency and differentiation pattern of patient 1 and patient 2 neurospheres compared with control cells (Fig. 6C). Annexin assays did not reveal difference in the proportions of apoptotic patient or control cells (Supplementary Material, Fig. S6).

Patient 1 and patient 2 cells examined after 5 weeks in differentiating conditions consisted of a mixture of neural precursors and differentiated neurons (Fig. 6A). Both patient neurons contained abundant distended LAMP1-positive vacuoles (Fig. 7A). Vacuole ultra-structure morphologies (Fig. 6D; Supplementary Material, S7) were reminiscent of those seen in undifferentiated patient iPSc (Fig. 2D). These storage vesicles were positive for LAMP1 and for the ganglioside GM3. The secondary accumulation if this glycosphingolipid is typical of MPSIII (Supplementary Material, Fig. S8). Western blot and staining showed increased LAMP1 and GM130 signals, compared with controls (Figs 3E and F and 7B). LAMP1-distended vesicles were co-stained with GM130 (Fig. 7C), and GM130 staining was more intense and broader with frequent extensions in neurites (Fig. 7D). Patient cells with neuronal morphology contained numerous abnormal Golgi complexes (Fig. 7E). Golgi complexes with disorganized morphology (51.2% in patients and 6.2% in controls) typically consisted of multiple short stacks that were not linked and often not aligned with respect to each other, associated with the accumulation of small vesicles. Storage vacuoles were frequently apposed at, or even connected to, the rims of fragmented ribbons. However, dysmorphic Golgi complexes collapsed in response to brefeldin A as efficiently as Golgi complexes in control cells, suggesting efficient vesicular trafficking in pre-Golgi compartments (Supplementary Material, Fig. S2B). These observations indicated prominent cell pathology in MPSIIIB human neurons.

DISCUSSION
We demonstrate that iPSc can be generated from the fibroblasts of children with MPSIIIB, a fatal LSD with major CNS involvement. NAGLU deficiency affected the proliferation of emerging iPSc clones, necessitating compensation of the genetic defect through enzyme replacement for efficient isolation. In contrast with parental patient fibroblasts, isolated iPSc rapidly exhibited prominent cell pathology once exogenous enzyme supply was halted. Disease expression did not
Human Molecular Genetics, 2011, Vol. 20, No. 18

impair iPSc survival and differentiation, allowing efficient isolation of affected neural stem cells and differentiated neurons. Whereas cell pathology was modest in floating neurosphere cultures, gene expression profiling revealed changes affecting multiple pathways, consistent with alterations of cell interactions with the environment. Neural stem cell adhesion and differentiation were accompanied by the installation of severe cell pathology consisting of intracellular vacuoles and severe disorganization of Golgi complex structure. The examination of patients’ pluripotent stem cells and their neural and neuronal progenies revealed for the first time the existence of Golgi defects in a lysosomal disorder.

Patient iPSc required compensation of or adaptation to enzyme deficiency for proliferation. Fibroblast reprogramming led to the emergence of iPSc clones with equal frequencies in patient and control fibroblasts. However, whereas clones emerging from normal fibroblasts rapidly proliferated in the presence of FGF2, emerging patient iPSc did not, suggesting inefficient response to this factor. This phenotype was reversed when feeder cells supplied the missing lysosomal enzyme. Enzyme replacement is currently a routine treatment for several MPSs (27). The exogenous enzyme clears GAGs, including HS, which accumulate as a consequence of the enzyme defect. HS proteoglycans act in concert with FGFs to transduce cell proliferation signals (23). In MPSs, the accumulation of partially degraded HS affects FGF2 signaling (28), as shown by impaired proliferation and survival of multipotent hematopoietic progenitors in MPSI mice (29), or inefficient FGF2-dependent neurogenesis and reduced FGF1, FGF2 and FGF1 receptor mRNA expression in MPSIIIB mice (12). Reversion of the proliferation defect of MPSIIIB patient iPSc after exogenous enzyme supply suggested that impaired FGF signaling was due to the accumulation of partially degraded HS. Impaired FGF signaling affects major biological functions in pluripotent stem cells, including self-renewal, proliferation, survival, differentiation and adhesion (30).

Enzyme replacement is sufficient to prevent pathology in mucopolysaccharidoses, indicating that partially degraded proteoglycans induce disorders. Once patient iPSc had been established in the presence of NAGLU exogenous enzyme, they could be starved from this supply. Enzyme-starved iPSc, as well as their progenies, rapidly accumulated HS proteoglycans. They nevertheless survived and efficiently proliferated, suggesting that efficient response to FGF2, and possibly to other undefined growth-survival factors, was now achieved despite HS accumulation. This observation is important as it indicates that modified biological functions identified in chronically deficient MPSIIIB cells may result from adaptive processes turned on to survive HS proteoglycan degradation defect.

Post-mortem studies of affected patient tissues (15,17) as well as pathology studies in MPSIIIB mice (18,19) showed predominant storage lesions in brain cells. These observations are consistent with the clinical expression of the disease, which predominates in the CNS. They are also consistent with mild or absent disease-related phenotype in patient skin fibroblasts, despite significant HS accumulation. Examination of human iPS-derived neurons, the cell type most relevant to clinical manifestation, therefore appears the best approach to investigate human MPSIIIB cell pathology. We show that

Figure 5. Neural precursors derived from patient iPSc clones have a mild phenotype. Neural progenitors were co-cultured with fibroblasts and cultured for 2 weeks. (A) Control and patient neurospheres were dissociated into single-cell suspension and plated on polyornithine–laminin-coated cover slips. Cells express nestin (in red) 1 day after adhesion. A representative image of one differentiated patient 1 clone is shown. Scale bar: 100 μm. (B) One representative flow cytometry diagram is shown and the bar chart indicates mean ± SEM of independent determinations in control (NS-C) and patient (NS-P) cells. Neuronal progenitors were stained with anti-GFAP antibody. One representative flow cytometry diagram is shown and the bar chart indicates mean ± SEM of independent determinations in control (n = 4) and patient (n = 5) neural cells. Difference is not significant (Mann–Whitney test). (C) Control (NS-C), patient 1 and patient 2 neurosphere pellets were processed for electron microscopy. High magnification images show representative images of Golgi complexes (GC). In few patient 2 cells (NS-P2), cisternae are interrupted and not well-aligned. Similar observations were made in patient 1 cells. N, nucleus. Scale bars: 0.5 μm.
Figure 6. Storage vesicles accumulate in patient differentiated neurons. Cultures of control or patient neurons were obtained from dissociated neurospheres and plated on polyornithine–laminin-coated cover slips. (A) Differentiated neurons positive for βIII-tubulin (in green) and neural precursors positive for nestin (in red) were observed after 3 weeks in control (DN-C), patient 1 (DN-P1) and patient 2 (not shown) cells. Representative images are shown. All control and patient iPSc clones differentiated with the same efficiencies. Scale bar: 100 μm. (B) (Left panels) MAP2-positive or βIII-tubulin-positive neurons (in green) expressing the GABA transporter VGAT (in red) or the glutamatic transport VGLUT (in red) were observed after 5 weeks in all neurosphere cultures. (Right panels) iPSc-derived MAP2-positive neurons (in green) express the mature neuronal marker synaptophysin (in red). Scale bar: 10 μm. (C) Equivalent amounts of MAP2 mRNAs were detected by qRT-PCR using amplification of ARPO mRNAs as a reference at days 0 (D0), 15 (D15), 21 (D21) and 29 (D29) after plating, indicating comparable differentiation kinetics for control and patient neurons. Data are means ± SEM (n = 3). (D) Cultures were processed for ultra-structural analysis 5 weeks after adhesion. Low magnification views (left panels, upper row) showed multiple intracellular vesicles in patient 1 (left) but not in control (right) cells with neuronal morphologies. Scale bars: 2 μm. High magnifications (left panels, medium and bottom rows) revealed intraluminal electro-dense fibrillar (medium left), granular (medium middle) or multilamellar (medium right, bottom) materials in patient 1 and patient 2 cells. (Two right panels) A distended vesicle (lower panel) in a long cell process (upper panel) that can be identified as a neuronal prolongation is shown. N, nucleus; StV, storage vesicle. Scale bars: 0.5 μm. See also Supplementary Material, Figure S7, showing a low magnification composite view of the same long process.
Figure 7. Golgi complex alterations in patient neurons. Control, patient 1 and patient 2 neurons derived from each iPSc clone were examined after 5 weeks of

Golgi complex alterations in patient neurons. Control, patient 1 and

distended vesicles. Scale bars: 10 μm. (B) High magnification of confocal views show more abundant distended LAMP1 vesicles and altered GM130 staining pattern in patient1 and patient 2 neurons compared with control (DN-C). Representative images of patient 1 neurons (DN-P1) are shown. Scale bars: 10 μm. (C) High magnification confocal view of a doubly stained neuron. Deconvoluted images without (left) or with (right) iso-surface treatment show contiguous staining for LAMP1 and GM130 in vesicle-limiting membranes (arrow). Scale bars: 2 μm. (D) The localization of GM130 signal with respect to the nucleus was analyzed in control and patient neurons. Left pictures are examples of low magnification Apotome views of neurons doubly stained for βIII-tubulin and GM130. Scale bar: 10 μm. The diagram in the right panel indicates the numbers of GM130-positive elements according to their distance from the nucleus in control (n = 78) and patient neurons (n = 91). Data show a broader dispersion of GM130 signals in patient than in control neurons. Neurons were randomly chosen for scoring (three independent experiments). (E) Golgi complex morphology was analyzed using electron microscopy in cells with a neuronal morphology. A typical Golgi complex in a control cell is shown on the left. Partially (middle picture) or fully (right picture) fragmented Golgi complexes in patients' cells are shown. Similar observations were made in patient 2. GC, Golgi complex; N, nucleus; StV, storage vesicle. Scale bar: 0.5 μm. The bar diagram on the right indicates the proportion of Golgi complex with normal (A), partially fragmented (B) or totally fragmented (C) morphology scored in control (n = 32) and patient (n = 80) randomly chosen cells.
HS proteoglycans are major determinants of how the cells sense environmental cues bound to ECM. As co-receptors for cell adhesion, cell proliferation and fate determination molecules and other extracellular signals, they collaborate with ‘primary’ receptors to control signaling. HS proteoglycans are endocytosed and fragmented into oligosaccharides by heparanases in early endosomes. In the absence of further degradation in lysosomes, as in NAGLU-deficient cells, oligosaccharides accumulate in intracellular compartments and are released in the environment, where they bind ECM proteins and modify the bioavailability of multiple signaling molecules. Changes in cell–matrix interactions resulting from this modified environment likely affect cell identification and responses to environmental cues important for adhesion, migration, process extension, polarization and fate specification (43–45). Modified control of the extra-cellular signal-regulated pathway, which is activated by FGFs and controls Golgi remodeling through the phosphorylation of GRASP65, might account for the observed disorganization of Golgi architecture (46). Interestingly, mild expression of this phenotype in floating neurospheres may result from less stringent dependence of non-adherent cells on matrix-bound cues, compared with adherent iPSC or differentiating neural cells. Similarly, matrix-bound cues important for iPSC and neural cells may be largely irrelevant for fibroblasts.

Previous gene expression profiling studies in MPSIIIB mouse tissues showed upregulation of genes related to lysosomal functions and inflammation (47–49). We were surprised that genes coding for lysosomal proteins, or proteins carrying mannose-6-phosphate residues (26), were either unchanged in fibroblasts accumulating HS or downregulated in patient neural stem cells. These observations do not support the hypothesis that deficient lysosomal functions induced lysosomes proliferation (50). In contrast, changes in the expression of genes related to Golgi functions in patient neural stem cells were consistent with the observed modifications of Golgi architecture.

Is MPSIIIB a developmental disease? Whereas cell defects identified in patient iPSC could impinge on their differentiation capacity, assays performed in several paradigms indicated that they behave like control iPSC, giving rise to differentiated cell progeny with similar efficiency, kinetics and pattern. These results appear consistent with the absence of clinical manifestations of the disease during fetal life and at birth in affected children (51,52) and animal models (19,53). However, these observations are not sufficient to conclude that stem cells behave normally in MPSIIIB. Indeed, the teratoma formation assay, which showed normal patient iPSC differentiation, was performed in recipient mice that are able to complement the enzymatic defect of patient iPSC. In affected humans, the CNS is presumably protected against HS proteoglycan toxicity until birth through the delivery of a maternal enzyme across the blood–brain barrier, as suggested by blood–brain barrier permeability to mannose-6-phosphate bearing molecules until birth in mice (54). Although EB formation and neural stem cell differentiation paradigms showed apparently normal behavior of patient iPSC in the absence of enzyme complementation, the accuracy of these in vitro assays is clearly not sufficient to detect subtle deviations from normal differentiation pattern, as they may occur in vivo. Considering the central role of matrix–cell interactions, cell migration, neurogenesis and neutriogenesis in post-natal CNS development, developmental damage may occur in MPSIIIB children. This possibility has major implications with regard to treatments aimed at restoring normal brain plasticity and cognition in affected children. Very early in life, reduction of HS burden is presumably the best situation for clinical success.

MATERIALS AND METHODS

iPSC generation and differentiation

Skin fibroblast cultures from two MPSIIIB patients and one non-affected volunteer were obtained from the Centre de Ressources Biologiques in Lyon (France) after approval by the competent authorities. A statement of biological samples was made according to French laws formulated by the Ministère de la Recherche et au Comité de Protection des Personnes Ile de France (reference DC-2009-1067). Fibroblasts were grown in DMEM Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma). iPSC clones were generated as previously described (55). Retrovirus plasmids carrying human cDNA coding for OCT4, SOX2, KLF4 and c-MYC were used (Addgene plasmids 17217, 17218, 17219, 17220). After VSV-G-pseudotyped retrovirus vector production, 5 × 10⁴ cells for MPSIIIB fibroblasts and 10⁵ cells for control fibroblasts were transduced twice with the retrovirus vectors in the presence of sulfate protamine (5 mg/ml, Sigma). Clones with well-defined hES-like morphology were observed 3 weeks after gene transfer. They were selected and manually picked 1 or 2 weeks later. iPSC clones were maintained on iMEF feeder layers in the following medium (iPSC medium): DMEM/F12 (Invitrogen) containing 20% KnockOut Serum Replacement (Invitrogen), 10 ng/ml of FG2 (Miltenyi Biotech, Paris, France), 100 µM nonessential amino acids (Invitrogen) and 100 µM 2-mercaptoethanol (Invitrogen). Cultures were passaged every 5–10 days either manually or enzymatically with collagenase type IV (1 mg/ml) (Invitrogen). MPSIIIB iPSC clones were isolated and cultured on iMEF transduced with a lentivirus vector encoding human NAGLU (NAGLU-iMEF) (11). NAGLU activities in cell supernatants were increased 4.3-fold in transduced cells. To culture iPSC clones without iMEF, iPSC clones were transferred onto Matrigel™-coated wells (BD Biosciences) in mTeSR™1 medium (Stem Cell Technologies). iPSC clones were passaged either manually or enzymatically with dispase (2 mg/ml, Invitrogen). The iPSC(IMR90)-4 cell clone (56) (WiCell Research Institute Madison, WA, USA) was also cultured on Matrigel™-coated wells in mTeSR™1 medium. For EB differentiation, iPSC clones were collected as small clusters after collagenase treatment. Clusters were resuspended in iPSC medium without FG2. Ten days later, EBs were plated on polyornithine (20 µg/ml) and laminin (10 µg/ml)-coated cover slips and incubated for 8 additional days. For neural differentiation, neurospheres were generated as previously described (31). To induce neural differentiation, neurospheres were dissociated into single-cell suspension with Accumax (PA Laboratories, Linz, Austria) and 2 × 10⁶ cells were plated onto polyornithine–laminin-coated cover slips in DMEM/F12 supplemented with 2% B27 (Invitrogen). To quantify plating efficiencies, cells were fixed with 4%
paraformaldehyde (PFA) and stained with DAPI. At least 200 nuclei were counted per cell type (n = 3 experiments). Equivalent plating efficiencies were calculated between control and patient cells.

**Immunocytochemistry and AP staining**

AP staining was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma). For immunocytochemistry, cells were fixed in 4% PFA for 20 min at room temperature. After washing with phosphate buffered saline (PBS, Invitrogen), cells were treated with PBS containing 2% normal goat serum (Invitrogen), 1% bovine serum albumin (Sigma) and saponin (0.01%, Sigma) or Triton X-100 (0.1%, Sigma) for 1 h at room temperature. Primary antibodies included mouse monoclonal anti-AFP (IgG2a, 1:200, Chemicon), anti-α-SMA (IgG2a, 1:200, Chemicon), anti-βIII-tubulin (IgG2a, 1:500, Eurogentec, Berkeley, CA, USA), anti-HS (IgM, 1:200, clone HepSS-1 kindly provided by Seikagaku Biobusiness Corporation, Tokyo, Japan), anti-GM3 (IgM, 1:100, kindly provided by Seikagaku Biobusiness Corporation), anti-LAMP1 (IgG1, 1:200, SouthernBiotech), anti-MAP2 (IgG1, 1:500, Chemicon), anti-O4 (IgM, 1:200, Stem Cell Technology), anti-SSEA4 (IgG3, 1:100, Chemicon), anti-SSEA1 (IgM, 1:100, Chemicon), anti-TRA1-60 (IgM, 1:100, Chemicon), anti-β-actin (IgG2b, 1:400, Synaptic System, Goettingen, Germany), rabbit anti-GM130 (1:500, Sigma), rabbit anti-NANOG (1:100, Abcam, Cambridge, UK), rabbit anti-nestin (1:400, Chemicon), rabbit anti-VGLUT (1:1000, Synaptic System), rabbit anti-synaptophysin (1:1000, Synaptic System), rabbit anti-giantin (1:500, Covance, Princeton, NJ, USA), rabbit anti-NG2 (1:700, Chemicon). Secondary antibodies were Alexa488 goat anti-mouse IgG1 (1:2000, Invitrogen), Alexa555 goat anti-mouse IgG2a (1:2000, Invitrogen), Alexa488 goat anti-mouse IgG2a (1:500, Invitrogen), Cy3 goat anti-mouse IgG3 (1:200, Jackson ImmunoResearch), rhodamine goat anti-mouse IgM (1:200, Millipore), FITC goat anti-mouse IgM (1:100, Jackson ImmunoResearch), biotin goat anti-mouse IgG1 (1:500, Jackson ImmunoResearch), Alexa488 goat anti-rabbit (1:1000, Invitrogen), Alexa555 goat anti-rabbit (1:1000, Invitrogen), Alexa647 streptavidin (1:500, Molecular Probes). Nuclei were stained with 1 mg/ml Hoechst H33342 (Invitrogen) and slides were mounted with Fluormount G (SouthernBiotech). Images were acquired either with an Axioplan 2 imaging optic microscope equipped with Apotome and AxioCam TR camera controlled by the AxioVision software (Zeiss, LE Pecq, France), or with an SP5 confocal system (Leica, Reuil-Malmaison, France). Confocal images were deconvoluted with the Huygens software (Svi, Hilversum, The Netherlands). Deconvolved 3D images were constructed using the Imaris x64 software (Bitplan, Zurich, Switzerland).

**Western blotting**

Cells were homogenized in lysis buffer [0.1% SDS, 1% NP-40, 0.2% deoxycholate, 0.15 M NaCl, 50 mM Tris, pH 7.8, and a protease inhibitor cocktail (Roche)], triturated and centrifuged at 12 000g for 20 min at 4°C. Cell lysates (10 µg) were separated by electrophoresis on 7% SDS–polyacrylamide gel (Invitrogen) and transferred to a nitrocellulose membrane. Signals were revealed with anti-LAMP1 (1:1000, SouthernBiotech), anti-GM130 (1:200, IgG1, clone 35, BD Biosciences) and anti-actin (1:2000, Sigma) antibodies followed by appropriate horseradish peroxidase-coupled secondary antibodies (1:10 000, Amersham) and enhanced chemiluminescence (Pierce). Signal intensities were measured with the LAS-1000CH Luminescent photofilm LTD system, piloted by the IR-LAS-Pro software (Fuji).

**Flow cytometry analysis and apoptosis**

Cells were fixed in 2% PFA and incubated for 1 h either with an anti-GM130 antibody (1:100) or with an isotypic mAb as a negative control in PBS containing 1% fetal calf serum. Secondary antibody used was an Alexa488 anti-mouse IgG1 (Invitrogen). Apoptosis was analyzed with the Vybrant Apoptosis Assay Kit (Invitrogen) according to the manufacturer’s protocol.

**GAG dosage**

Neurospheres were cultured by centrifugation and overnight lysed in acetate buffer (100 mM sodium acetate, pH 5, containing 5 mM cystein, 5 mM EDTA) supplemented with papain 3% (Sigma) at 65°C. After centrifugation, sulfated GAGs were quantified in supernatants with the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor) according to the manufacturer’s protocol.

**Electron microscopy**

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide for 1 h. Cells were then dehydrated through increasing ethanol baths, and embedded in Epon 812. Ultra-thin sections (70 nm) were prepared using a Reichert Ultracut S Wild M3z microtome (Leica) and contrasted with uranyl acetate and lead citrate. Ultra-structural analyses were performed on a JEOL 1200EXII transmission electron microscope (JEOL) equipped with an Eloise Megaview camera controlled by the Analysis Pro 3.1 software (Eloise).

**MPSIIIB genotyping and karyotyping**

Genotyping of MPSIIIB mutations was performed by PCR amplification and sequencing of genomic DNA (Supplementary Material, Table S3). Karyotyping was performed by Pasteur-Cerba (Cergy-Pontoise, France). Analyses were performed on the following clones: C2 (passage 8), P2.3 (passage 11), P1.1 (passage 10), P1.3 (passage 16), P2.2 (passage 16), P2.3 (passage 18).
with ‘RT-PCR’ primers (Supplementary Material, Table S3). qRT-PCR was performed in a Model 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA) with 100 ng of cDNA and the TaqMan PCR Master Mix (according to Applied Biosystems procedures). Design of qRT-PCR primers (Supplementary Material, Table S3) was done using the Primer Express software.

**Bisulfite sequencing**

Genomic DNA was treated with EpiTech Bisulfite according to the manufacturer’s recommendations (Qiagen). The promoter region of the human OCT4 gene was amplified by PCR. The PCR products were subcloned into pCR2-TOPO (Invitrogen). Ten clones of each sample were verified by sequencing (Eurofins MWG Operon). Primers’ ‘bisulfite sequencing’ are listed in Supplementary Material, Table S3.

**Teratoma formation**

Approximately $2 \times 10^6$ dissociated iPSc were injected intramuscularly in Rag $\gamma C^{-/-}$ mice. Six weeks after injection, teratomas were dissected, fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

**Affymetrix exon array hybridization and data analysis**

Fibroblasts from patient 2 were exposed to lentivirus vectors coding for GFP (HS+), or to two vectors coding for GFP and NAGLU, respectively (HS−). GFP expression and NAGLU activity were verified in three independent cultures of each type, from which 100 ng of total RNA was isolated, providing three independent samples for each condition. After amplification and labeling with Affymetrix reagents, targets were hybridized to Affymetrix Human Genome U-133 Plus 2.0 chips (47 000 transcripts, 38 500 genes). For analyses, a third synthetic sample (C1–3) was generated from the two control arrays (Eurofins MWG Operon). Primers’ ‘bisulfite sequencing’ are listed in Supplementary Material, Table S3.

**Statistical analyses**

Statistics were performed using the SPSS software (SPSS).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.
Induced pluripotent stem cell lines derived from human somatic cells. 


